DELHI PHARMACEUTICAL SCIENCES & RESEARCH UNIVERSITY

(Incorporating DIPSAR as Constituent College as per DPSRU Act)

First DSRC Report
for
Doctor of Philosophy (Ph.D.)

Govt. of N.C.T. of Delhi,

Pushp Vihar, Sector – III, M.B. Road,

New Delhi-110017

Website: www.dpsru.edu.in

(Approved by UGC under section 2f of UGC Act)

Accreditation by Life Sciences Sector Skill Development Council

DELHI PHARMACEUTICAL SCIENCES & RESEARCH UNIVERSITY

Research Scholar: Ashwini Kumar Mishra

Registration No.: 13/PhD/DPSRU/2019

Registration Date: 28th January 2022

PhD Supervisor: Prof. Pravat K. Sahoo

Project Title: FORMULATION AND DEVELOPMENT OF SILYMARIN AND PIPERINE LOADED NANOPARTICLES WITH NATURAL BIOENHANCER AGAINST PARACETAMOL INDUCED HEPATOTOXICITY MOUSE MODEL

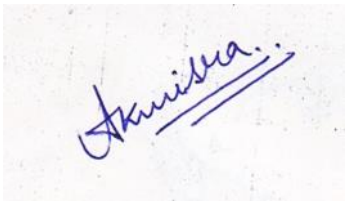
PART – A

PROFORMA FOR HALF-YEARLY PROGRESS REPORT OF REGISTERED RESEARCH SCHOLARS FOR THE PERIOD ENDING JANUARY 2020 PROGRESS REPORT FROM AUGUST 2019 TO JANUARY 2022

1. Name of Research Scholar: Ashwini Kumar Mishra
2. Correspondence Address: B 18 Krishna Park Deoli Road New Delhi
3. Contact No. 6394082143
4. Email: ashwinik757@gmail.com
5. Regn. No. 13/PhD/DPSRU/2019
6. Date of Regn. 13/08/2019
7. Semester Fee deposited**: Rs. 30385/- Vide R. No. PH/ADMISSION/DPSRU/11 Dated: 6/11/2017
8. Approved Topic of Research: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Acetaminophen Induced Hepatotoxicity Mouse Model
9. Nature of Fellowship, if any: NO
10. Details of leaves availed during the period under report, if any; Nil
11. *Research work done during the period:

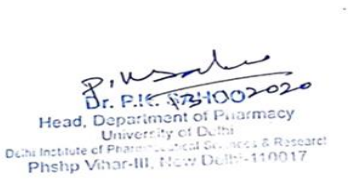
1. Evaluation of Preliminary Work
    - Objective
    - Methodology
    - Preliminary Results
2. Institutional Animal ethical committee approval

12. *Research papers published: Communicated
13. * Any other information not covered above: N/A



Date: (Name/Signature of the Research Scholar)

*Attach separate sheets, if required, for details. **Attach photocopies of University Cash Receipt as proof of semester fee deposited.



Countersignature of Supervisor

PART- B

REPORT OF THE DSRC

Name of the research Scholar ASHWINI KUMAR MISHRA.....

Registration No.....13/PhD./DPSRU./2019.....

Conduct of the Research Scholar.....

Progress Report: (Annexure)

Overall Assessment of the progress and specific comments:

The title of Research work is approved. Literature work has been carried out. Various techniques of nano-formulation has been proposed / explored.

20.2.2020

(Expert 1) (Expert 2) (Supervisor) (Co-Supervisor)

NA

Date: 20.02.2020

Introduction

Silymarin, a mixture of flavonolignan including silybin A, silybin B, isosilybin A, isosilybin B, silychristin, silydianin and flavonoid polyphenolic compounds extractable from milk thistle (*Silybum marianum*) seeds, has anti-oxidant, anti-inflammatory, anti-cancer and anti-viral activities potentially useful in the treatment of several liver disorders, such as chronic liver diseases, cirrhosis and hepatocellular carcinoma*(Vargas-Mendoza et al., 2014)*. However, although clinical trials have proved that Silymarin is safe at high doses (>1500 mg/day) in humans, the bulky multi-ring structure exhibits limiting factors such as poor aqueous solubility (<50 mcg/mL), low bioavailability and poor intestinal absorption. Hence, the clinical role of silymarin is limited. To solve this problem, an array of methods are available in the literature such as formation of silybin–phospholipid complex, solid dispersions, dextrose modified bilosomes, encapsulated liposomes, and self-micro emulsifying drug delivery system, have been used to boost the rate of silymarin dissolution *(Di Costanzo et al., 2019)* Compared with raw Silymarin, the dissolution and bioavailability of treated silymarin has been improved.

Nevertheless, in various Silymarin preparations, many disadvantages were encountered. There was a large particle size and poor stability in the silybin-phospholipid complex and the silybin solid dispersions. The encapsulated liposomes and dextrose modified bilosomes endured a complex preparation method and asserted poor stability. Furthermore, the self-emulsifying drug delivery system addressed inconvenience in storage and transport.

Our work aimed at using natural products as a bioenhancer (piperine – a known inhibitor of hepatic and intestinal glucoronidation inhibitor) together with silymarin as a dual drug loaded nanoparticles to enhance its bioavailability by either increasing its water solubility, improving its permeability or impeding its metabolism.

Literature Review

- ***Peterson et al. (2019)***, studied the advantages linked with the bioenhancers are non-toxicity, efficacy at low concentrations, convenient to formulate with the drug, increased drug uptake and absorption, and finally, synergizing the drug activity. Bioenhancers enhance the bioavailability of nutraceuticals by intervening via several pathways, including gastrointestinal action to improve absorption or by altering the cycle of drug metabolism.
- ***Javed et al. (2018),*** investigated various combinations of Silymarin with natural bioenhancers such as Lysergol (L), Piperine (P) and Fulvic acid (FA) were prepared and

their hepatoprotective activities were evaluated against carbon tetrachloride (CCl4) induced hepatotoxicity in animal model.

Rationale of Proposed Work

As per the data put up by the Institute of Liver and Biliary Sciences, established by the Government of the National Capital Territory (NCT) of Delhi, Hepatic diseases are severe health problems since, when used in overdose, various pharmaceuticals, xenobiotics, environmental / chemical toxins, other natural products and nutritional supplements tend to cause hepatotoxicity. Silymarin has tremendous potential for the treatment of various liver disorders. Medical success for Silymarin is, however, constrained by very low aqueous solubility and associated biopharmaceutical limitations. These factors necessitate the incorporation of silymarin into a form that can augment its bioavailability. An effective solution to this problem could be to deliver Silymarin along with natural bioenhancer-Piperine as a dual-drug loaded delivery molecule (nanoparticle) in place of conventional dosage form. Silymarin-piperine nanoparticles (SPNs) will be prepared by various techniques and to study the effect of manufacturing parameters which will identify suitable technique. SPNs will be strongly protective against hepatic damage for which test will be perform on a paracetamol overdose hepatotoxicity model.

Objectives

- To prepare Silymarin-piperine dual-drug loaded nanoparticles using different techniques.
- To study the effects of various manufacturing parameters on Silymarin-piperine nanoparticles (SPNs)
- In-vitro release assay of nanoparticles (SPNs) as performance test.
- To explore hepatoprotective and antioxidant effects of nanoparticles (SPNs) against Paracetamol **(N-acetyl-ρ-aminophenol, APAP)** induced hepatotoxicity in mouse.

Plan of Work

A. Selection and procurement of drugs and polymers.
Preformulation studies and preparation of standard curve.
Preparation of Placebo/Drug nanoparticles.

B. Optimization of nanoparticles by response surface methodology
Characterization of optimized nanoparticles

C. In-vitro drug release and in-vivo hepatoprotective studies

D. Data Analysis and compilation, research paper and thesis writing

Methodology

1. **Preformulation studies**
    i. **Physicochemical properties (Identification tests and Solubility testing)**
    ii. **Spectral analysis: UV, DSC, FTIR**
    iii. **Partition coefficient**
    iv. **Melting point**
2. **Formulation and development of dual-drug loaded nanoparticles**
3. **Optimization of SPNs using experimental design**
4. **Characterization of prepared SPNs**
5. ***In-vitro* release assay as performance test**
6. ***In-vivo* hepatoprotective and antioxidant study protocol**
7. **Statistical evaluation of data**
8. **Compilation and submission of data, summary, results, discussion and conclusion.**

Expected Outcomes

- A novel nanoparticles delivery device for Silymarin-piperine was successfully developed in Poloxamer 188, Poloxamer 407, Eudragit 100 ® and protective properties will be identified against hepatotoxicity induced by APAP.
- A novel approach to liver toxicity treatment **by synergistic hepatoprotective activity and increased silymarin bioavailability** by using natural products as bioenhancers.
- Advancement in Oral drug delivery.
- **Increased** SPNs-induced rapid regeneration of **hepatic Glutathione** levels
- Compared with crude silymarin, **nanoparticle-assisted** improved **solubilization** and permeability results in enhanced bioavailability can be observed of Silymarin.
- If worthwhile effects are revealed in preclinical studies **clinical studies can also be designed.**

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1. Di Costanzo A, Angelico R. Formulation Strategies for Enhancing the Bioavailability of Silymarin: The State of the Art. Molecules. 2019 Jan; 24(11):2155.

2. Javed S, Ahsan W, Kohli K. Pharmacological influences of natural products as bioenhancers of silymarin against carbon tetrachloride-induced hepatotoxicity in rats. Clinical Phytoscience. 2018 Dec; 4(1):1-9.
3. Peterson B, Weyers M, Steenekamp JH, Steyn JD, Gouws C, Hamman JH. Drug bioavailability enhancing agents of natural origin (bioenhancers) that modulate drug membrane permeation and pre-systemic metabolism. Pharmaceutics. 2019 Jan; 11(1):33.
4. Vargas-Mendoza N, Madrigal-Santillán E, Morales-González Á, Esquivel-Soto J, Esquivel-Chirino C, y González-Rubio MG, Gayosso-de-Lucio JA, Morales-González JA. Hepatoprotective effect of silymarin. World journal of hepatology. 2014 Mar 27; 6(3):144.

Workshop/Conferences:

1. **Ashwini Kumar Mishra,** P.K Sahoo, "Antidiabetic effect of Trigonella Foenum graceum L. granules in low-dose streptozotocin induced diabetic rats", 7th International Congress Of Society For Ethno-pharmacology SFEC-2020, Jamia Hamdard, New Delhi,15th-17th February 2020.
2. Attended the workshop "Rheometer and Its Applications in Pharmacy", organized by Anton Paar held in Delhi Pharmaceutical Sciences and Research University, New Delhi, 27th August 2019.

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Second DSRC Report
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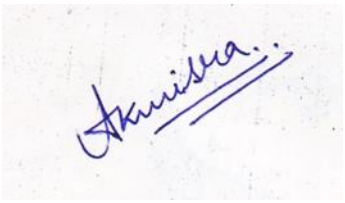

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Countersignature of Supervisor

PART - B

REPORT OF THE DSRC

Name of the Research Scholar: Ashwini Kumar Mishra

Registration no.: 13/PhD/DPSRU/2019

Conduct of the Research Scholar:

Progress Report: (Annexure I)

Overall Assessment of the progress and specific comments of the Supervisor:

Experts1-

Mr. Ashwini Kumar Mishra has achieved all the proposed objectives of the research work. Pharmacodynamics and Pharmacokinetics studies has been carried out. He has published two research and one review articles of the proposed research work. Therefore it is recommended that Mr. Ashwini Kumar Mishra may be submit his thesis.

6.6.23
(Kaketan Kohli)

Date: 12.6.2023

Experts 2-

(Supervisor)
Prof. (Dr.): Pravat K. Sahoo
Director, DIPSAR

Annexure-I

SECOND PROGRSES REPORT

(Project Synopsis)

Evaluation of Preliminary Work

I. Title of Research: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Acetaminophen Induced Hepatotoxicity Mouse Model

II. Introduction

Preformulation studies: Preformulation is described as a phase of the research and development process during which physical, chemical and mechanical properties of drug substance is characterized, in order to develop stable safe and effective dosage form. It is the early phase in the discovery which aid in selection of drug molecule to enter in development process.

A thorough understanding of physical, chemical, and mechanical property of drug ultimately provide a rational for formulation design or support the need for molecular modification or merely confirm that there are no significant barriers to the compound's development.

The objectives of the Preformulation are

1. To established the physicochemical characteristics of the drug substance.
2. To developed method of validation for drug
3. To established compatibility of drug with different excipients.

The studies which are conducted as basic Preformulation studies depending on the type of dosage form and the type of drug molecule are as follow-

1. **Characterization of the selected Drug**

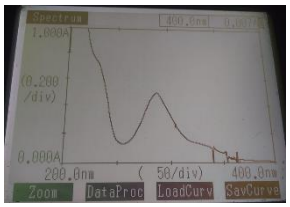
Physical Characterization of Silymarin

Parameter	Values
Color	Dark Yellow
State	Powder
Melting Point	155-170℃
Partition Coefficient log P oct/pH7.4	1.71
Solubility	78.9mcg/ml
Solubility	61.1mcg/ml

Physical Characterization of Piperine

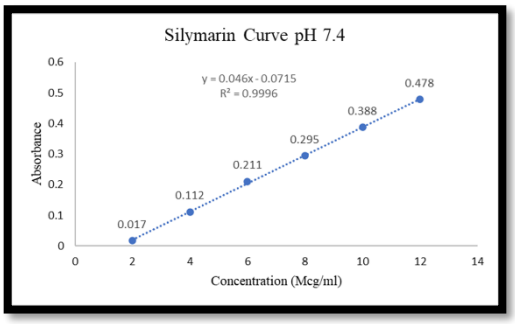
Parameter	Values
Color	White to Yellow
State	Amorphous Powder
Melting Point	126-130℃
Partition Coefficient log P oct/pH7.4	2.46
Solubility	119.8mcg/ml
Solubility	54 mcg/ml

Absorbance Maxima of Silymarin



The UV spectra obtained from validated method had its λmax peak at 287.7 nm in phosphate buffer pH 7.4.

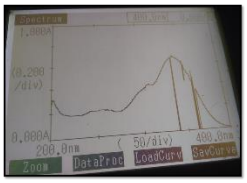
Calibration Curve of Silymarin



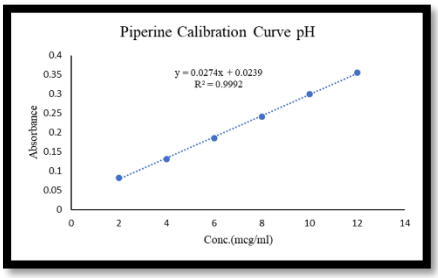
Conc. (Mcg/ml)	Absorbance
2	0.017
4	0.112
6	0.211
8	0.295
10	0.388
12	0.478

Absorbance Maxima of Piperine

The UV spectra obtained from validated method had its λmax peak at 341.5 nm in phosphate buffer pH 7.4.



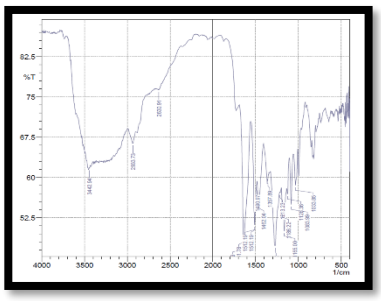
Calibration Curve of Piperine in Phosphate Buffer 7.4



Conc. (Mcg/ml)	Absorbance
2	0.083
4	0.131
6	0.186
8	0.241
10	0.299
12	0.355

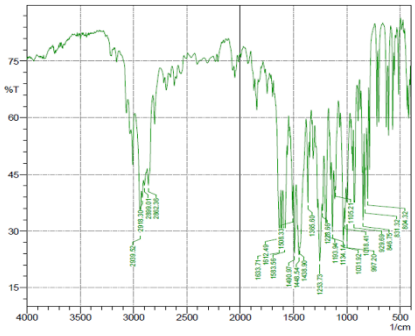
FTIR Spectra of Silymarin

FTIR spectrum of Silymarin and mixture of excipients was obtained using FTIR spectrophotometer by spread as a thin layer on potassium bromide cell and then scanned between 4000 - 500 cm -1 The result shows that the peaks of Silymarin and Physical Mixture from the mixture have similar peaks that means it doesn't have any interaction with excipients.



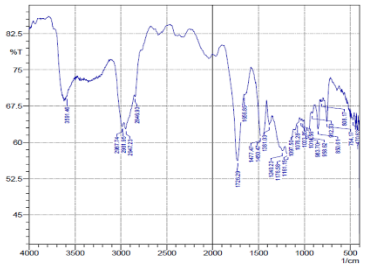
FTIR Spectra of Piperine

FTIR spectrum of Piperine and mixture of excipients was obtained using FTIR spectrophotometer by spread as a thin layer on potassium bromide cell and then scanned between 4000 - 500 cm -1 The result shows that the peaks of Piperine and Physical Mixture from the mixture have similar peaks that means it doesn't have any interaction with excipients.



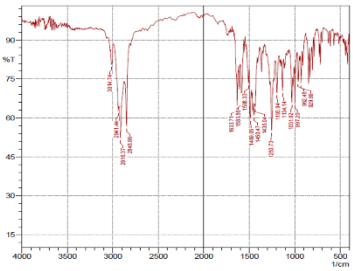
FTIR Spectra of Eudragit RL 100

FTIR spectrum of Eudragit RL100 was obtained using FTIR spectrophotometer by spread as a thin layer on potassium bromide cell and then scanned between 4000 - 500 cm $^{-1}$



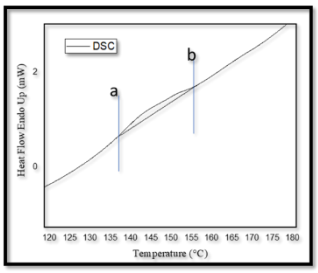
FTIR Spectra of Physical Mixture

FTIR spectrum of Physical Mixture FTIR spectrophotometer by spread as a thin layer on potassium bromide cell and then scanned between 4000 - 500 cm $^{-1}$

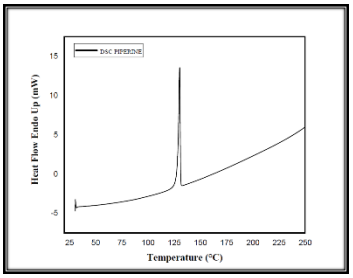


Differential scanning colorimetry (DSC) of Silymarin

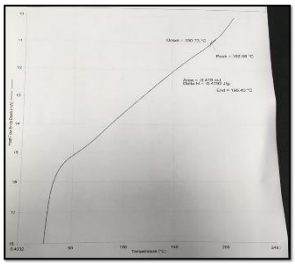
The Point a to b indicates the exothermic peak. The quenched material has a well-defined Tg indicating significant amorphous structure which rearranges on heating to a crystalline structure before melting at about 200°C.



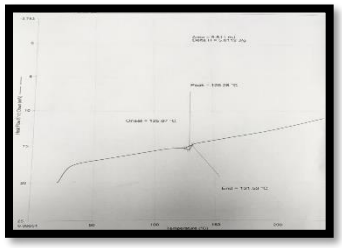
Differential scanning colorimetry (DSC) of Piperine



Differential scanning colorimetry (DSC) of ERL 100

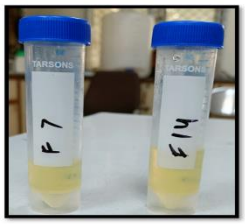


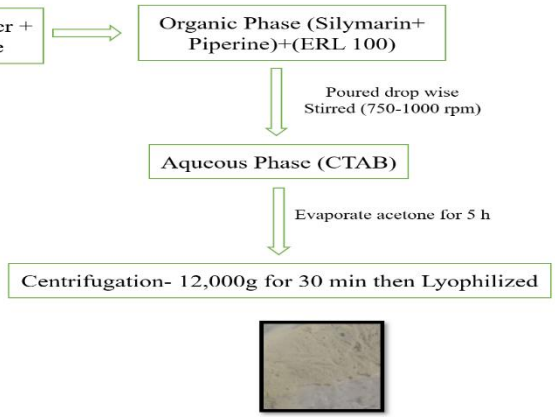
Differential scanning colorimetry (DSC) of Physical Mixture



Formulation and development of dual-drug loaded nanoparticles

Nanoprecipitation method was adopted in this work in order to prepare a dual-drug loaded nanoparticles comprising of Silymarin and Piperine.




Drugs + Polymer + 30ml acetone
Organic Phase (Silymarin+ Piperine)+(ERL 100)
Poured drop wise
Stirred (750-1000 rpm)
Aqueous Phase (CTAB)
Evaporate acetone for 5 h
Centrifugation- 12,000g for 30 min then Lyophilized

Preparation Of Nanoparticles

S.NO	Ingredients	Uses
1	Silymarin	Drug
2	Piperine	Drug
3	Acetone	Organic phase (Solvent)
4	CTAB	Surfactant
5	Eudragit RL 100	Polymer
6	Distilled water	Aqueous Phase

Rnn	Silymarin (in mg)	Piperine (in mg)	Acetone (in ml)	CTAB (in mg)	Eudragit RL 100 (in mg)	Water (in ml)
F1	50	25	10	300	200	30
F2	50	25	10	200	200	30
F3	50	25	10	200	100	30
F4	50	25	10	200	200	30
F5	50	25	10	100	200	30
F6	50	25	10	200	200	30
F7	50	25	10	300	300	30
F8	50	25	10	300	100	30
F9	50	25	10	100	200	30
F10	50	25	10	200	300	30
F11	50	25	10	100	100	30
F12	50	25	10	100	300	30
F13	50	25	10	200	100	30
F14	50	25	10	200	200	30
F15	50	25	10	200	300	30
F16	50	25	10	300	200	30
F17	50	25	10	200	200	30

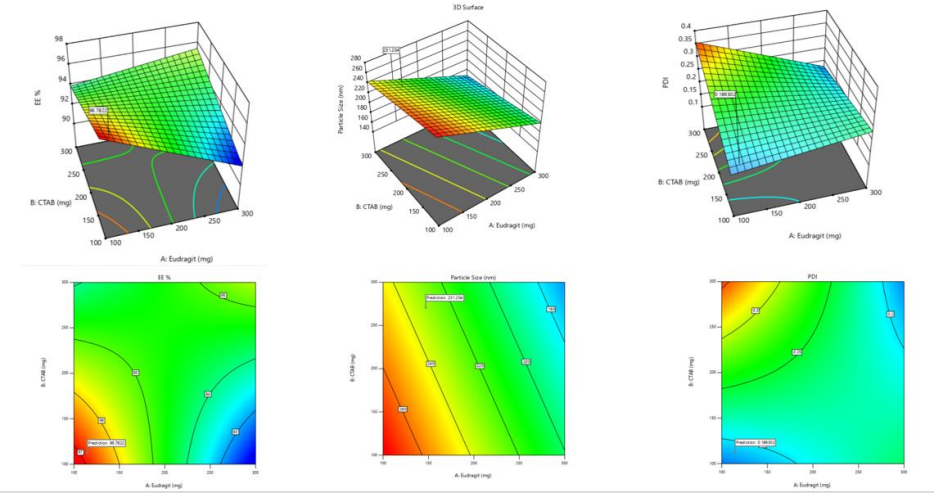
Optimization of Formulation

		Variables				
		Independent variables		Dependent variable		
Runs	Factor A: ERL 100	Factor B: CTAB	Factor C: Stirring Speed (RPM)	Response Y1: % EE	Response Y2: Zeta size	Response Y3: PDI
1	200	300	500	94.859	229.6	0.369
2	200	200	750	95.708	354.3	0.245
3	100	200	500	93.096	166.8	0.259
4	200	200	750	96.288	294.3	0.246
5	200	100	500	96.444	254.6	0.237
6	200	200	750	94.993	219.7	0.298
7	**300**	**300**	**750**	**97.025**	**156.6**	**0.342**
8	100	300	750	93.475	250.9	0.343
9	200	100	1000	93.453	222.9	0.196
10	300	200	1000	92.225	221.9	0.198
11	100	100	750	96.746	247.4	0.149
12	300	100	750	93.353	202.6	0.23
13	100	200	1000	96.589	173.9	0.281
14	**200**	**200**	**750**	**95.92**	**150.4**	**0.238**
15	300	200	500	96.79	156.6	0.259
16	200	300	1000	93.431	192.5	0.242
17	200	200	750	94.346	195.3	0.247

Analysis for Responses (Zeta size, PDI and Entrapment efficiency) Fit modal summery for responses

Responses	Sources	Sequential P-value	Lack of Fit P-value	Adjusted R^2	Predicted R^2	Adequate precision	
Zeta size	Quadratic	0.0173	0.8381	0.9213	0.2838	7.277	Suggested
PDI	Quadratic	0.0014	0.3959	0.9522	0.4248	11.615	Suggested
% EE	Quadratic	< 0.0022	0.4512	0.9273	0.4789	10.2429	Suggested

3-D surface Plot For % EE, SIZE and PDI



Characterization of Optimized Formulation

Silymarin-piperine nanoparticles (S-P-Nps) were prepared and optimized using design of experiment Software by box-benkhen method. Characterization of S-P-Nps was performed and results are as follows:

- The entrapment efficiency of optimized S-P-Nps was found to be 97.025 %.
- The shape and size of the developed formulation was observed with TEM. Particles of 150-400 nm were observed. The result shows that particles were spherical and approximately 160 nm in size.
- PDI value of optimized formulation indicates minimal agglomeration and observed that PDI and size increases on increasing concentration of CTAB (cetyltrimethylammonium bromide).
- The **SEM and TEM images** of nanoparticles revealed the morphology and distribution of particles. The shape and size of the developed formulation was observed with TEM. Particles of 120-400 nm were observed. The result shows that particles were spherical and approximately 156 nm in size.

Future Objectives

- In-vitro release study of Silymarin-Piperine NPs
- Antioxidant Activity of Silymarin-Piperine NPs
- Hepatoprotective activity of Silymarin-Piperine NPs on paracetamol induced liver toxicity in mice
- Pharmacokinetic study of Silymarin-Piperine NPs

DELHI PHARMACEUTICAL SCIENCES & RESEARCH UNIVERSITY

(Incorporating DIPSAR as Constituent College as per DPSRU Act)

THIRD DSRC Report
for
Doctor of Philosophy (Ph.D.)

Govt. of N.C.T. of Delhi,
Pushp Vihar, Sector – III, M.B. Road,
New Delhi-110017
Website: www.dpsru.edu.in

(Approved by UGC under section 2f of UGC Act)
Accreditation by Life Sciences Sector Skill Development Council

DELHI PHARMACEUTICAL SCIENCES & RESEARCH UNIVERSITY

Research Scholar: Ashwini Kumar Mishra

Registration No.: 13/PhD/DPSRU/2019

Registration Date: 19/10/2019

PhD Supervisor: Prof. Pravat K. Sahoo

Project Title: FORMULATION AND DEVELOPMENT OF SILYMARIN AND PIPERINE LOADED NANOPARTICLES WITH NATURAL BIOENHANCER AGAINST PARACETAMOL INDUCED HEPATOTOXICITY MOUSE MODEL

PART – A

PROFORMA FOR HALF-YEARLY PROGRESS REPORT OF REGISTERED RESEARCH SCHOLARS FOR THE PERIOD ENDING JANUARY 2020 PROGRESS REPORT FROM AUGUST 2019 TO JANUARY 2023

1. Name of Research Scholar: Ashwini Kumar Mishra
2. Correspondence Address: B 18 Krishna Park Deoli Road New Delhi
3. Contact No. 6394082143
4. Email: ashwinik757@gmail.com
5. Regn. No. 13/PhD/DPSRU/2019
6. Date of Regn. 13/08/2019
7. Semester Fee deposited**: Rs. 30385/- Vide R. No. PH/ADMISSION/DPSRU/11 Dated: 6/11/2017
8. Approved Topic of Research: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Acetaminophen Induced Hepatotoxicity Mouse Model
9. Nature of Fellowship, if any: NO
10. Details of leaves availed during the period under report, if any; Nil
11. *Research work done during the period:

1. Evaluation of Preliminary Work
    - Objective
    - Methodology
    - Preliminary Results
2. Institutional Animal ethical committee approval

12. *Research papers published: Communicated
13. * Any other information not covered above: N/A

Date: (Name/Signature of the Research Scholar)

*Attach separate sheets, if required, for details. **Attach photocopies of University Cash Receipt as proof of semester fee deposited.

Countersignature of Supervisor

PART - B

REPORT OF THE DSRC

Name of the Research Scholar: Ashwini Kumar Mishra

Registration no.: 13/PhD/DPSRU/2019

Conduct of the Research Scholar:

Progress Report: (Annexure I)

Overall Assessment of the progress and specific comments of the Supervisor:

Experts1- Kanchan Kohli

In-vitro release studies have been carried out by using Dialysis membrane no. 60 (High media) and compared with marketed formulation. Pharmacodynamic studies have been done by an animal model as per literature. Antioxidant studies have also been carried out. Remaining work: Pharmaco-Kinetic studies.

Experts 2-

(Supervisor)
Prof. (Dr.): Pravat K. Sahoo
Director, DIPSAR

Date: 14.2.2023

Page | 4

Ashwini Kumar Mishra-13/Ph.D/DPSRU/2019

Annexure-I

FOURTH PROGRSES REPORT
(Project Synopsis)

Title of Research: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Acetaminophen Induced Hepatotoxicity Mouse Model

Result & Discussion

A. Formulation of Silymarin-piperine NPs (SL-P NPs) and DOE-based optimization

The SL-P NPs were prepared in different batches in which concentrations of Eudragit RL100 and CTAB were varied at different rpm. The preparation method aids to synthesize NPs in a size range of 220-421 nm, having a PDI range between 0.138 to 0.331.

Particle Size Analysis

The average size range of NPs varied from 220-421 nm, which was influenced by changing the concentration of ERL, CTAB, and stirring speed. Upon analyzing the data statistically, the model proposed (quadratic) was significant with a value of $p < .0001$, F-value (85.94), and a coefficient of correlation i.e., $R^2 = 0.991$ respectively.

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Entrapment efficiency (%EE)

EE of SL-P NPs was found to be in a range of 82.22-96.43%. Adjusted and predicted values of R2 obtained were 0.989 and 0.980 respectively. Now, it was founded that the positive impact of ERL concentration was seen in the organic phase concerning the incorporation of sufficient drug payload resulting in a lower level of %EE.

Zeta Potential

The zeta potential of SL-P NPs was determined to be +15.6± 2.17 mV, indicating the polymeric nanoparticles stability. In nanosized drug delivery systems, zeta potential is an essential indication for the stability of nano dispersion because nanoparticles with larger negative or positive zeta potential display stronger repulsive forces, resulting in enhanced system stability.

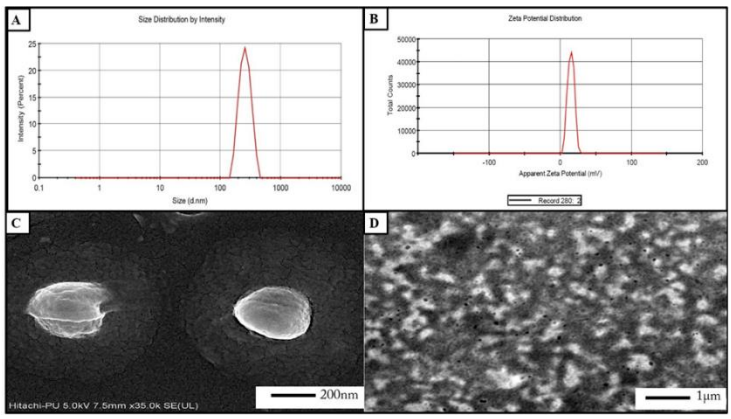
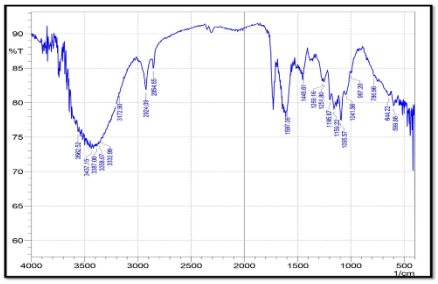


Fig.1. Particle size distribution (A), Zeta potential (B), SEM (C) and TEM(D) of SL-P NPs

B. Characterization of Optimized Formulation Silymarin-piperine NPs

FTIR analysis

Freeze-dried optimized SL-P NPs **(Fig.2.)** revealed mostly the Eudragit RL 100 absorption peaks, with a few overlapping drug peaks. It may be determined that no significant drug-polymer interaction occurred within the nanoparticles.



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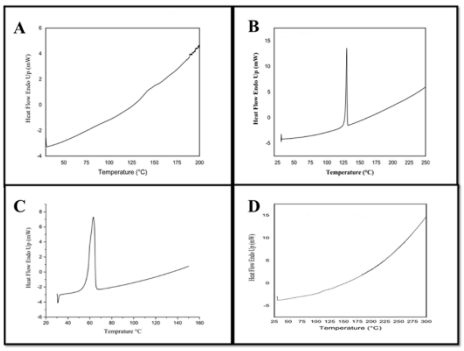

Fig.3. DSC thermograms of Silymarin(A), Piperine (B), Physical Mixture (C) and SL-P NPs (D).

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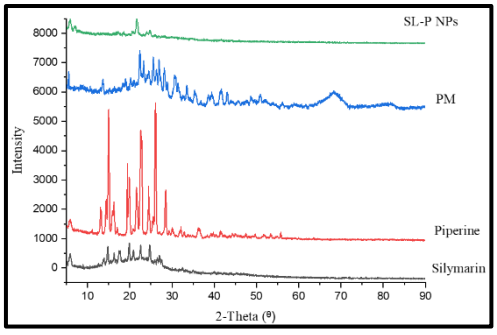

Fig.4. XRD diffraction analysis of Silymarin, Piperine, Physical mixture (PM) and optimized SL-P NPs

SEM and TEM studies

The surface morphology of optimized SL-P NPs was represented with the help of an SEM image **(Fig.1.C).** Optimized nanoparticles were seeming to be nearly spherical with a smooth surface and dispersed evenly with minimal aggregation. The TEM picture **(Fig.1.D)** clearly shows that most of the emulsion particles in the optimized formulation were less than 250 nm in size and spherical.

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The raw silymarin, raw piperine, and Silybon-70 showed immediate release profiles of 94± 2.51%, 72.55 ± 3.22%, and 99.91± 3.48% in 9hr, 12 hrs., and 3 hrs. respectively. But in the case of optimized SL-P NPs, the release profile was found to be slow and sustained with 96.16± 2.87% in 24hrs. For each model, the coefficient of correlation (R2) was computed along with the kinetics constraints. Based on R2 best-fitted model was found to be Weibull for SL-P NPs (R2= 0.998), Korsmeyer-Peppas for raw Silymarin, and raw piperine (R2= 0.997, 0.935), and zero-order for Silybon-70 (R2= 0.995).

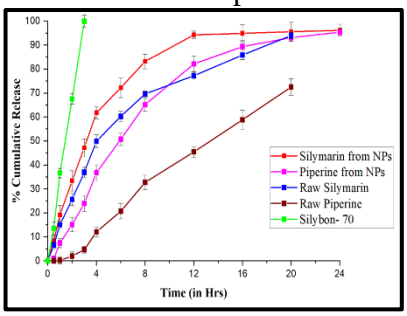

Fig.5. Drug release pattern of Silymarin and Piperine from optimized nanoparticles loaded with SL-P, raw Silymarin, raw Piperine, marketed formulation (Silybon-70)

C. Antioxidant potential of SL-Piperine NPs

The results showed that the antioxidant effect of SL-P NPs was higher as compared to raw drug and Vitamin E as the piperine and SL combinedly give a potentiating effect to the prepared NPs.

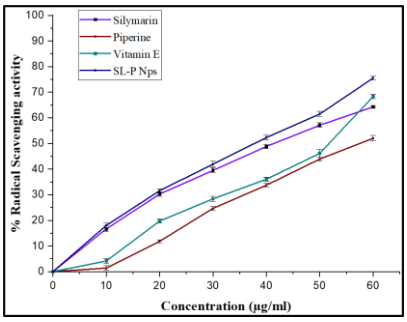

Fig.6. Comparison of DPPH % Radical Scavenging activity of Silymarin, Piperine, Vitamin E and SL-P NPs

D. In-vivo hepatotoxicity and biochemical analysis

When compared to normal control mice (Group A), high dose N-acetyl-p-amino-phenol intoxication (Group B) and empty nanoparticles along with N-acetyl-p-amino-phenol administration (Group C) significantly elevated SGOT, SGPT, ALKP and serum bilirubin concentrations. N-acetyl-p-amino-phenol also lowered the total protein in serum, serum GSH as well as tissue GSH levels in groups B and C. On the other hand, the results observed are different where the biochemical parameters (SGOT, SGPT, ALKP, and serum bilirubin concentrations) were substantially decreased in N-acetyl-p-amino-phenol intoxicated mice with market formulation (Group D), SL-Piperine (Group E) and SL-P NPs (Group F) treatment compared to Group B and C. It should be noted that these parameters were decreased

remarkably when N-acetyl-p-amino-phenol -treated mice were treated with SL-P NPs (Group F) compared to market formulation (Group D) and SL-Piperine (Group E). The combination of SL-P NPs provided stronger protection from N-acetyl-p-amino-phenol -induced hepatotoxic injury than either alone in the case of market product and SL-Piperine.

Future Objectives

- Histopathology of Silymarin-Piperine NPs
- HPLC based pharmacokinetic study of Silymarin-Piperine NPs
- Stability study data of prepared formulation

References

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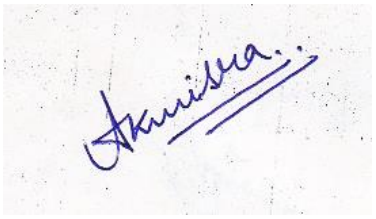
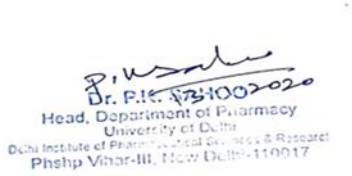


Signature

Ashwini Kumar Mishra

(PhD. Scholar)


Head, Department of Pharmacy
University of Delhi
Delhi Institute of Pharmaceutical Sciences & Research
Pushp Vihar-III, New Delhi-110017

Signature

Prof. (Dr.) P.K Sahoo

(Supervisor)

DELHI PHARMACEUTICAL SCIENCES & RESEARCH UNIVERSITY

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Fourth DSRC Report
for
Doctor of Philosophy (Ph.D.)

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(Approved by UGC under section 2f of UGC Act)

Accreditation by Life Sciences Sector Skill Development Council

Ashwini Kumar Mishra-13/Ph.D/DPSRU/2019

DELHI PHARMACEUTICAL SCIENCES & RESEARCH UNIVERSITY

Research Scholar: Ashwini Kumar Mishra

Registration No.: 13/PhD/DPSRU/2019

Registration Date: 19/10/2019

PhD Supervisor: Prof. Pravat K. Sahoo

Project Title: FORMULATION AND DEVELOPMENT OF SILYMARIN AND PIPERINE LOADED NANOPARTICLES WITH NATURAL BIOENHANCER AGAINST PARACETAMOL INDUCED HEPATOTOXICITY MOUSE MODEL

PART – A

PROFORMA FOR HALF-YEARLY PROGRESS REPORT OF REGISTERED RESEARCH SCHOLARS FOR THE PERIOD ENDING JANUARY 2020 PROGRESS REPORT FROM AUGUST 2019 TO JANUARY 2023

1. Name of Research Scholar: Ashwini Kumar Mishra
2. Correspondence Address: B 18 Krishna Park Deoli Road New Delhi
3. Contact No. 6394082143
4. Email: ashwinik757@gmail.com
5. Regn. No. 13/PhD/DPSRU/2019
6. Date of Regn. 13/08/2019
7. Semester Fee deposited**: Rs. 30385/- Vide R. No. PH/ADMISSION/DPSRU/11 Dated: 6/11/2017
8. Approved Topic of Research: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Paracetamol Induced Hepatotoxicity Mouse Model
9. Nature of Fellowship, if any: NO
10. Details of leaves availed during the period under report, if any; Nil
11. *Research work done during the period:

1. Evaluation of Preliminary Work
    - Objective
    - Methodology
    - Preliminary Results
2. Institutional Animal ethical committee approval

12. *Research papers published: Communicated
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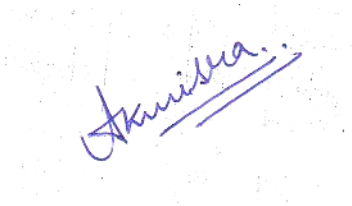

Date: (Name/Signature of the Research Scholar)

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Countersignature of Supervisor

PART - B

REPORT OF THE DSRC

Name of the Research Scholar: Ashwini Kumar Mishra

Registration no.: 13/PhD/DPSRU/2019

Conduct of the Research Scholar:

Progress Report: (Annexure I)

Overall Assessment of the progress and specific comments of the Supervisor:

Experts1-

Mr. Ashwini Kumar Mishra has achieved all the proposed objectives of the research work. Pharmacodynamics and Pharmacokinetics studies has been carried out. He has published two research and one review articles of the proposed research work. Therefore it is recommended that Mr. Ashwini Kumar Mishra may be submit his thesis.

(Kaketan Kohli)

Date: 12.6.2023

Experts 2-

(Supervisor)
Prof. (Dr.): Pravat K. Sahoo
Director, DIPSAR

Ashwini Kumar Mishra-13/Ph.D/DPSRU/2019

Annexure-I

FOURTH PROGRESS REPORT
(Project Synopsis)

Title of Research: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Paracetamol Induced Hepatotoxicity Mouse Model

Result & Discussion

A. Formulation of Silymarin-piperine NPs (SL-P NPs) and DOE-based optimization

The SL-P NPs were prepared in different batches in which concentrations of Eudragit RL100 and CTAB were varied at different rpm. The preparation method aids in synthesizing NPs in a size range of 220-421 nm, having a PDI range between 0.138 and 0.331.

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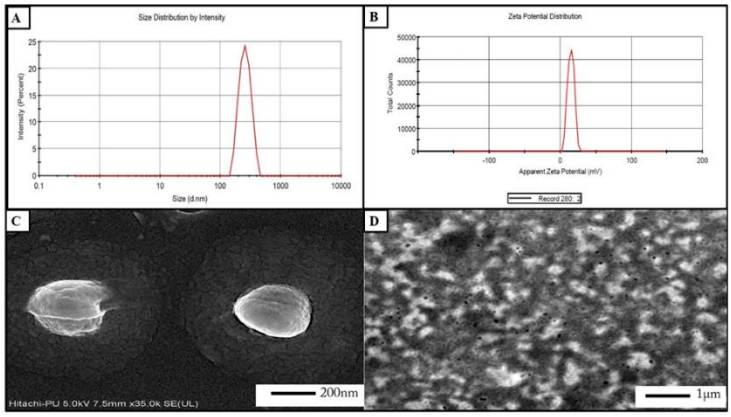

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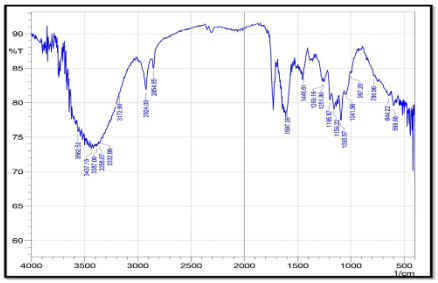

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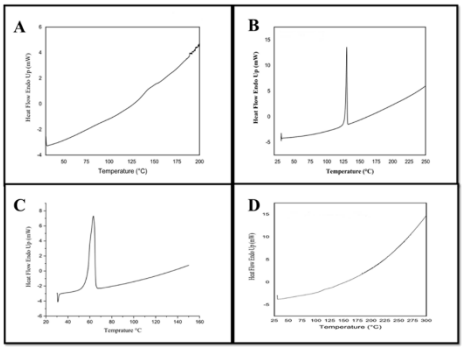
DSC Studies



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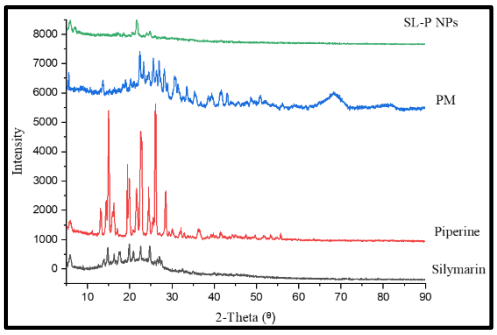

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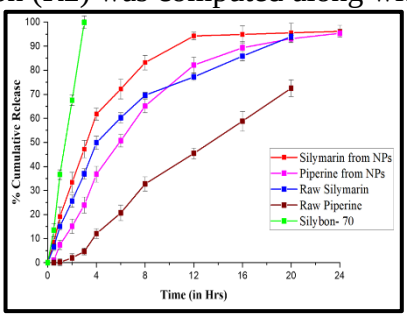

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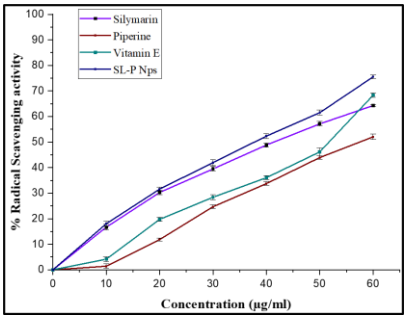

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D. In-vivo hepatotoxicity and biochemical analysis

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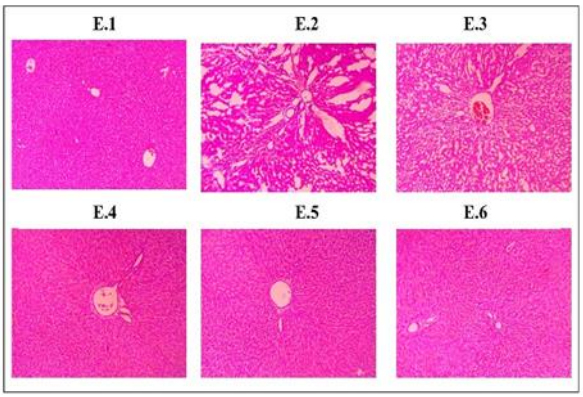
E. Histopathology

Histopathological examinations of mice liver portrayed normally organized hepatocytes formed radiating hepatic cords surrounding the central vein, with normal lobular architecture including blood sinusoids, portal structures, and no visible alteration in a tissue section from



control group A was detected **(Fig.E.1).** On contrary to group A, significant alterations were detected in the hepatic parenchyma, including disruption of the normal hepatic cord organization, congested central vein, and sinusoids due to focal necrosis, a lathered vacuolated cytoplasm, ballooning injury and evident degenerative changes characterized by severe nuclear pyknosis in sections of Paracetamol treated mice group B and C **(Fig.E.2, E.3).** Macrophages migrated towards the necrotic zone and were also observed. The findings agreed with those found in the literature. Furthermore, neither the marketed tablet formulation treated group nor plain silymarin-piperine saline suspension appreciably alleviated the Paracetamol -induced deterioration of liver tissue architecture. Photomicrographs **(Fig.E.4, E.5)** demonstrated non-significant protection of hepatocytes against oxidative stress and hepatotoxins. There was dilatation in the hepatic sinusoids, which was correlated with infiltration of inflammatory cells and disseminated liver cell macrophages between the injured hepatocytes. This might be owing to the inadequate or decreased bioavailability of silymarin to liver cells in both formulations. Interestingly, SL-P NPs treated mice group significantly restored normal hepatic architecture **(Fig.E.6).** Furthermore, the designed nanoparticulate formulation treatment of SL-P strongly protected mice against hepatotoxins and oxidative damage caused liver injury as the results showed minimal necrosis and absence of foamy vacuolated cytoplasm in these groups as compared to Paracetamol treated group. Hence, it would be a worthy statement toward the current research study that the increased antioxidant potential, enhanced bioavailability, and improved solubility were achieved when silymarin was used in combination with piperine incorporated in nano formulation. It was observed that with better hepatoprotective potential inhibition of progression of liver diseases would be achieved.

F. HPLC based pharmacokinetic study of Silymarin-Piperine NPs

Novel Gradient Flow HPLC system was developed for Pharmacokinetic evaluation of developed formulation.

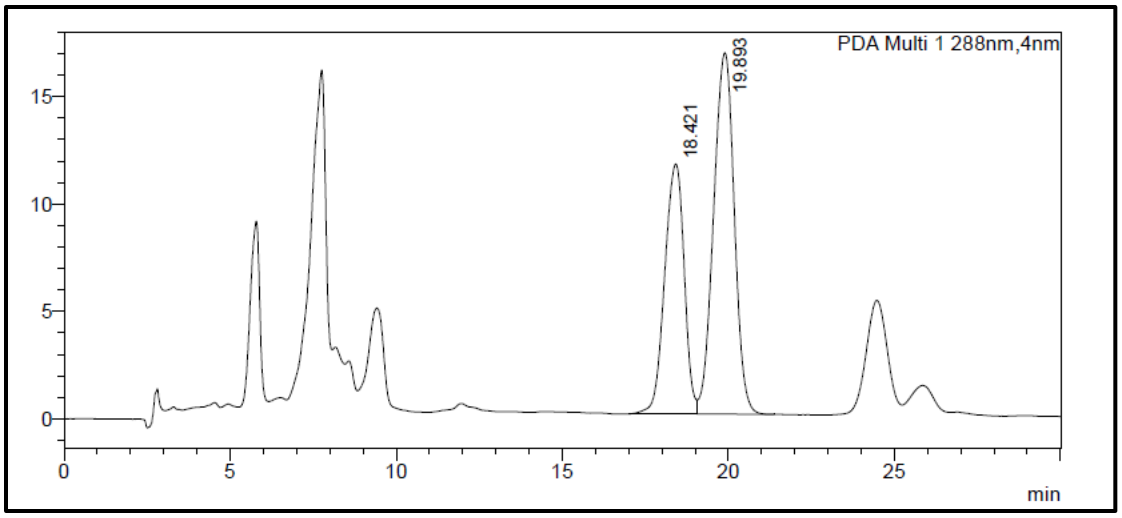
For Silymarin

Solvent A: - pH 2.3 Phosphate Buffer

Solvent B: - Methanol

Ratio of Solvent system- solvent A (60 %): Solvent B (40%)

Injection volume= 20 µl, oven temp: - 35 ^{o}c, R_t found at 18.42 and 19.89 minute (Silibinin A and B)



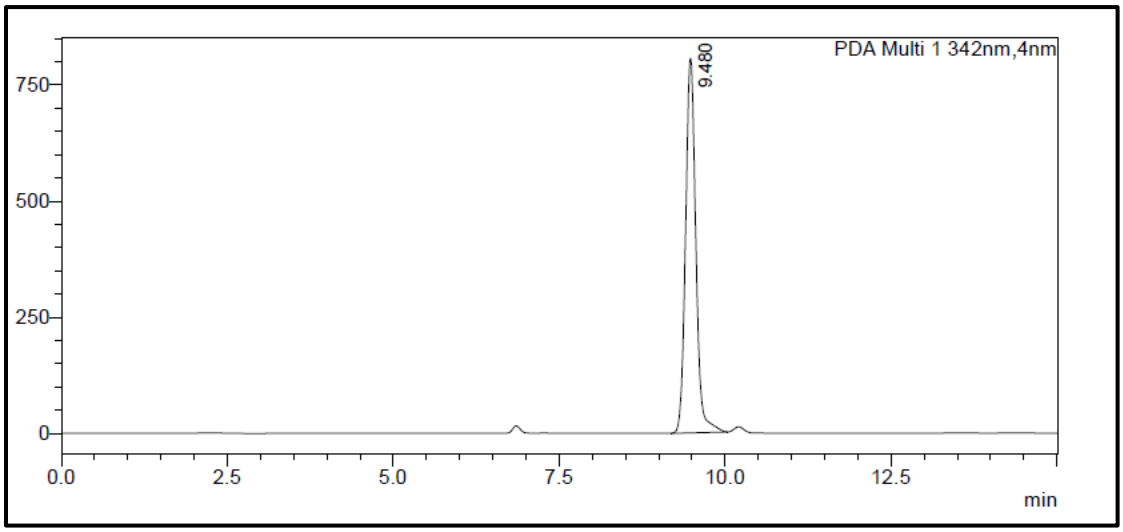
For Piperine

Solvent A: - Acetonitrile

Solvent B: - Water with 0.5ml formic acid

Ratio of Solvent system- solvent A (70 %): Solvent B (30%)

Injection volume= 20 µl, oven temp: - 35 ^{o}c, R_t found at 9.4 minute.



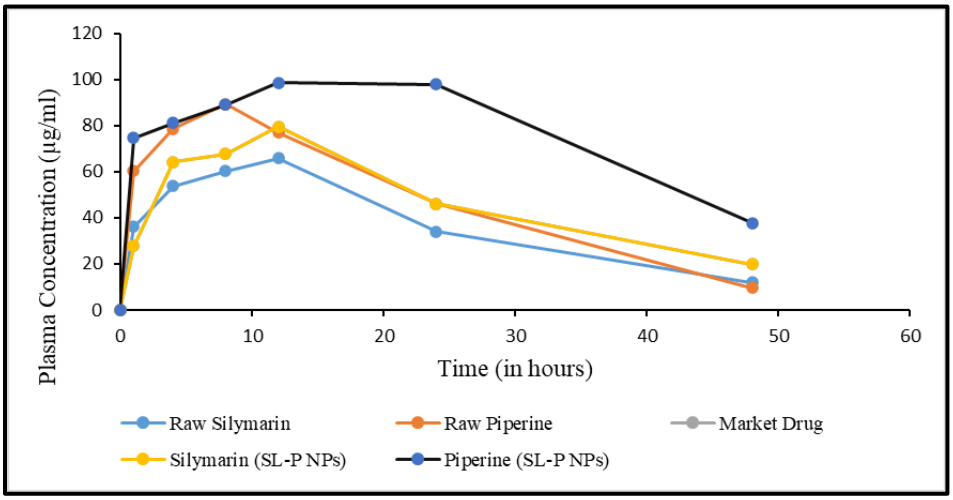
After oral administration of treatment, blood samples were withdrawn from each treatment rat group. The serum was isolated using cooling centrifuge, and Silymarin and piperine was isolated from serum by liquid-liquid isolation technique and analysis of drug was done by developed HPLC method. The different pharmacokinetic parameters in 100mg + 20mg combination (Silymarin + Piperine) dose Silymarin group, Piperine group, 100 mg marketed

Silybon tablet and SL-P NPs group were detected by using PK Solver software. Table given below shows Pharmacokinetic parameters in blood and drug concentration in liver-

Group (n=5)	**C_{max} (µg/ml)**	**T_{max} (h)**	**$T_{1/2}$ (h)**	**AUC 0-t (µg/ml*h)**	**Vz/F_obs (mg/kg)/(µg/ml)**	**Cl/F_obs (mg/kg)/(µg/ml)/h**
Raw Silymarin	65.97	12	14.7	1784.55	0.2609	0.0122
Raw Piperine	89.25	8	12.2	2313.93	0.0356	0.0020
Marketed Drug (Silybon-70)	79.65	12	18.27	2258.02	0.2366	0.0089
Silymarin from SL-P NPs	96.74	12	24.47	3249.16	0.1943	0.0055
Piperine from SL-P NPs	98.17	12	24.21	3796.51	0.034	0.0009

Figure given below Shows plasma concentration vs. time graph for Raw silymarin, Raw Piperine marketed Drug, Silymarin (SL-P NPs) and Piperine (SL-P NPs).

The observed results showed that the $t_{1/2}$ of developed formulation is approximately 24 hours as compared to marketed which sustains for 12 hours.



G. Stability study data of prepared formulation

The results of stability studies showed minimal variations in particle size, %EE, PDI and zeta potential during the storage of 180 days at stability conditions of 25 ±2℃ /RH of 60%±5% described in **Table G.1.** The results demonstrated that SL-P NPs were stable at 25 ±2℃ for 180 days with no significant change in particle size and entrapment efficiency. The results showed that PDI of the prepared formulation get increased whereas zeta potential get decreased over a periodically, this might be due to the nanoparticles begin to aggregate and the polymer begins to degrade at temperatures over 25℃, their stability quickly deteriorates. It can be concluded that the prepared SL-P NPs must be stored at temperature below 25℃ where they would remain stable in terms of PDI and zeta potential.

Time (in days)	Particle Size (nm)	% EE	PDI	Zeta Potential (mV)
Conditions- 25 ±2 ℃ /RH of 60%±5%.				
0	247.4± 4.5	95.92± 1.01	0.242± 0.002	+15.6± 2.17
30	250.9± 4.4	94.71± 0.25	0.196±0.004	+15.1± 2.17
90	286.5± 2.4	93.52± 0.16	0.414±0.002	+13.7±2.12
180	295.3± 2.8	91.67±0.95	0.685± 0.006	12.9±2.16

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Signature

Prof. (Dr.) P.K Sahoo

(Supervisor)

Executive summary for URAC for First DSRC Progress report

Research Scholar: Ashwini Kumar Mishra

Registration No.: 13/PhD/DPSRU/2019

Registration Date: 19th October 2019

Name of Supervisor: Prof. Pravat K. Sahoo

Project Title: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Acetaminophen Induced Hepatotoxicity Mouse Model

Introduction

Silymarin, a mixture of flavonolignan including silybin A, silybin B, isosilybin A, isosilybin B, silychristin, silydianin and flavonoid polyphenolic compounds extractable from milk thistle (*Silybum marianum*) seeds, has anti-oxidant, anti-inflammatory, anti-cancer and anti-viral activities potentially useful in the treatment of several liver disorders, such as chronic liver diseases, cirrhosis and hepatocellular carcinoma*(Vargas-Mendoza et al., 2014)*. However, although clinical trials have proved that Silymarin is safe at high doses (>1500 mg/day) in humans, the bulky multi-ring structure exhibits limiting factors such as poor aqueous solubility (<50 mcg/mL), low bioavailability and poor intestinal absorption. Hence, the clinical role of silymarin is limited. To solve this problem, an array of methods are available in the literature such as formation of silybin–phospholipid complex, solid dispersions, dextrose modified bilosomes, encapsulated liposomes, and self-micro emulsifying drug delivery system, have been used to boost the rate of silymarin dissolution *(Di Costanzo et al., 2019)* Compared with raw Silymarin, the dissolution and bioavailability of treated silymarin has been improved.

Nevertheless, in various Silymarin preparations, many disadvantages were encountered. There was a large particle size and poor stability in the silybin-phospholipid complex and the silybin solid dispersions. The encapsulated liposomes and dextrose modified bilosomes endured a complex preparation method and asserted poor stability. Furthermore, the self-emulsifying drug delivery system addressed inconvenience in storage and transport.

Our work aimed at using natural products as a bioenhancer (piperine – a known inhibitor of hepatic and intestinal glucoronidation inhibitor) together with silymarin as a dual drug

loaded nanoparticles to enhance its bioavailability by either increasing its water solubility, improving its permeability or impeding its metabolism.

Literature Review

- ***Peterson et al. (2019)***, studied the advantages linked with the bioenhancers are non-toxicity, efficacy at low concentrations, convenient to formulate with the drug, increased drug uptake and absorption, and finally, synergizing the drug activity. Bioenhancers enhance the bioavailability of nutraceuticals by intervening via several pathways, including gastrointestinal action to improve absorption or by altering the cycle of drug metabolism.
- ***Javed et al. (2018),*** investigated various combinations of Silymarin with natural bioenhancers such as Lysergol (L), Piperine (P) and Fulvic acid (FA) were prepared and their hepatoprotective activities were evaluated against carbon tetrachloride (CCl4) induced hepatotoxicity in animal model.

Rationale of Proposed Work

As per the data put up by the Institute of Liver and Biliary Sciences, established by the Government of the National Capital Territory (NCT) of Delhi, Hepatic diseases are severe health problems since, when used in overdose, various pharmaceuticals, xenobiotics, environmental / chemical toxins, other natural products and nutritional supplements tend to cause hepatotoxicity. Silymarin has tremendous potential for the treatment of various liver disorders. Medical success for Silymarin is, however, constrained by very low aqueous solubility and associated biopharmaceutical limitations. These factors necessitate the incorporation of silymarin into a form that can augment its bioavailability. An effective solution to this problem could be to deliver Silymarin along with natural bioenhancer-Piperine as a dual-drug loaded delivery molecule (nanoparticle) in place of conventional dosage form. Silymarin-piperine nanoparticles (SPNs) will be prepared by various techniques and to study the effect of manufacturing parameters which will identify suitable technique. SPNs will be strongly protective against hepatic damage for which test will be perform on a paracetamol overdose hepatotoxicity model.

Objectives

- To prepare Silymarin-piperine dual-drug loaded nanoparticles using different techniques.

- To study the effects of various manufacturing parameters on Silymarin-piperine nanoparticles (SPNs)
- In-vitro release assay of nanoparticles (SPNs) as performance test.
- To explore hepatoprotective and antioxidant effects of nanoparticles (SPNs) against Paracetamol **(N-acetyl-ρ-aminophenol, APAP)** induced hepatotoxicity in mouse.

Plan of Work

A. Selection and procurement of drugs and polymers.
Preformulation studies and preparation of standard curve.
Preparation of Placebo/Drug nanoparticles.

B. Optimization of nanoparticles by response surface methodology
Characterization of optimized nanoparticles

C. In-vitro drug release and in-vivo hepatoprotective studies

D. Data Analysis and compilation, research paper and thesis writing

Methodology

1. **Preformulation studies**
   i. **Physicochemical properties (Identification tests and Solubility testing)**
   ii. **Spectral analysis: UV, DSC, FTIR**
   iii. **Partition coefficient**
   iv. **Melting point**
2. **Formulation and development of dual-drug loaded nanoparticles**
3. **Optimization of SPNs using experimental design**
4. **Characterization of prepared SPNs**
5. ***In-vitro* release assay as performance test**
6. ***In-vivo* hepatoprotective and antioxidant study protocol**
7. **Statistical evaluation of data**
8. **Compilation and submission of data, summary, results, discussion and conclusion.**

Expected Outcomes

- A novel nanoparticles delivery device for Silymarin-piperine was successfully developed in Poloxamer 188, Poloxamer 407, Eudragit 100 ® and protective properties will be identified against hepatotoxicity induced by APAP.

- A novel approach to liver toxicity treatment **by synergistic hepatoprotective activity and increased silymarin bioavailability** by using natural products as bioenhancers.
- Advancement in Oral drug delivery.
- **Increased** SPNs-induced rapid regeneration of **hepatic Glutathione** levels
- Compared with crude silymarin, **nanoparticle-assisted** improved **solubilization** and permeability results in enhanced bioavailability can be observed of Silymarin.
- If worthwhile effects are revealed in preclinical studies **clinical studies can also be designed.**

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Recommendation of DSRC Committee: Work progress is satisfactory.

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Signature of Student **Signature of Supervisor(s)**

Executive summary for URAC for Second DSRC Progress report

Research Scholar: Ashwini Kumar Mishra

Registration No.: 13/PhD/DPSRU/2019

Registration Date: 19th October 2019

Name of Supervisor: Prof. Pravat K. Sahoo

Project Title: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Acetaminophen Induced Hepatotoxicity Mouse Model

Abstract

In present study, natural product as bioenhancer is being selected based on its mechanism of action: Piperine – a known inhibitor of hepatic and intestinal glucuronidation inhibitor. This study will present enhanced liver cell targeting efficacy, absorption with long-acting drug formulation and to stablish its preparation method, particularly to a high-efficacy with increased bioavailability and improved absorption of silymarin by the virtue of piperine which is known for its bio-enhancing property. The technique nanoprecipitation will be adopted in this work in order to prepare a dual-drug loaded nanoparticles comprising of Silymarin and Piperine (a natural bioenhancer) for high-efficacy, long-acting formulation of silymarin characteristic of high bioavailability and smooth in vivo targeted release. Piperine also have hepatoprotective activity which will provide synergistic potential to the drug formulation. Hepatoprotective and antioxidant potential of Silymarin-piperine nanoparticles (SPNs) will be explored against N-acetyl-ρ-aminophenol (APAP) induced hepatotoxicity in mouse. In short, the dual-drug loaded nanomedicine will provide enhanced bioavailability of Silymarin by Piperine as Bioenhancer along with promising efficacy and safety potential.

The following work has been done for 2nd DSRC:

1. Characterization of the selected Drugs
2. Formulation and development of dual-drug loaded nanoparticles
3. Optimization of Formulation
4. Characterization of Optimized Formulation including
    a. Entrapment efficiency of optimized Silymarin-piperine nanoparticles (S-P-Nps)

b. Surface morphology of the developed formulation was observed with SEM and TEM.
c. FTIR of optimized S-P-Nps
d. DSC of optimized S-P-Nps

The studies which are conducted as basic Preformulation studies depending on the type of dosage form and the type of drug molecule are as follow-

1. **Characterization of the selected Drug**

Physical Characterization of Silymarin

Parameter	Values
Color	Dark Yellow
State	Powder
Melting Point	155-170℃
Partition Coefficient log P oct/pH7.4	1.71
Solubility	78.9mcg/ml
Solubility	61.1mcg/ml

Physical Characterization of Piperine

Parameter	Values
Color	White to Yellow
State	Amorphous Powder
Melting Point	126-130℃
Partition Coefficient log P oct/pH7.4	2.46
Solubility	119.8mcg/ml
Solubility	54 mcg/ml

Silymarin-piperine nanoparticles (S-P-Nps) were prepared and optimized using design of experiment Software by box-benkhen method. Characterization of S-P-Nps was performed and results are as follows:

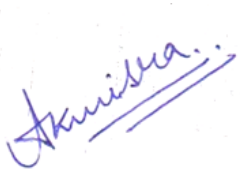
- The entrapment efficiency of optimized S-P-Nps was found to be 97.025 %.
- The shape and size of the developed formulation was observed with TEM. Particles of 150-400 nm were observed. The result shows that particles were spherical and approximately 160 nm in size.
- PDI value of optimized formulation indicates minimal agglomeration and observed that PDI and size increases on increasing concentration of CTAB (cetyltrimethylammonium bromide).

- The **SEM and TEM images** of nanoparticles revealed the morphology and distribution of particles. The shape and size of the developed formulation was observed with TEM. Particles of 120-400 nm were observed. The result shows that particles were spherical and approximately 156 nm in size.

Compliance of the earlier URAC recommendations: Compiled with.

- **Comment by URAC**: To clarify whether the efficacy of the targeted compound will be compared with that of standard compound.
- **Justification by student:** Standard Drug has to be compared with the nanoparticulate formulation for its bioavailability and solubility data profile and results are included in the DSRC report for all of the characterization done compared with standard compound.

Recommendation of DSRC Committee: Work progress is satisfactory.



Signature of Student **Signature of Supervisor(s)**

Executive Summary (3rd DSRC)

Research Scholar: Ashwini Kumar Mishra

Registration No.: 13/PhD/DPSRU/2019

Registration Date: 19th October 2019

Date of DSRC: 14 February 2023

Name of Supervisor: Prof. Pravat K. Sahoo

Project Title: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Acetaminophen Induced Hepatotoxicity Mouse Model

Abstract of work done (300-500 words, in structured manner with separate paragraphs for Introduction ending with Objectives; Methodology; Results; Conclusions and proposed further work with expected outcomes):

Abstract

In present study, natural product as bioenhancer is being selected based on its mechanism of action: Piperine – a known inhibitor of hepatic and intestinal glucuronidation inhibitor. This study will present enhanced liver cell targeting efficacy, absorption with long-acting drug formulation and to stablish its preparation method, particularly to a high-efficacy with increased bioavailability and improved absorption of silymarin by the virtue of piperine which is known for its bio-enhancing property. The technique nanoprecipitation will be adopted in this work in order to prepare a dual-drug loaded nanoparticles comprising of Silymarin and Piperine (a natural bioenhancer) for high-efficacy, long-acting formulation of silymarin characteristic of high bioavailability and smooth in vivo targeted release. Piperine also have hepatoprotective activity which will provide synergistic potential to the drug formulation. Hepatoprotective and antioxidant potential of Silymarin-piperine nanoparticles (SL-P NPs) will be explored against Acetaminophen induced hepatotoxicity in mouse. In short, the dual-drug loaded nanomedicine will provide enhanced bioavailability of Silymarin by Piperine as Bioenhancer along with promising efficacy and safety potential.

Results; Conclusions and proposed further work with expected outcomes:

The following work has been done for 3nd DSRC:

1. **Formulation and Optimization of SL-P NPs**

The SL-P NPs were prepared in different batches in which concentrations of Eudragit RL100 and CTAB were varied at different rpm. The preparation method aids to synthesize NPs in a size range of 220-421 nm, having a PDI range between 0.138 to 0.331.

2. **Characterization of Optimized SL-P NPs Formulation including**
    a. **Particle Size Analysis** -The average size range of NPs varied from 220-421 nm, which was influenced by changing the concentration of ERL, CTAB, and stirring speed.
    b. **Polydispersity index (PDI) analysis-** PDI range was found to be in limits of 0.138-0.331 and ANOVA was performed to show the factors. The p-value (0.0001) less than 0.05 denotes the model terms as significant.
    c. **Entrapment efficiency (%EE)-** EE of SL-P NPs was found to be in a range of 82.22-96.43%. Adjusted and predicted values of R^2 obtained were 0.989 and 0.980 respectively.
    d. Zeta Potential- The zeta potential of SL-P NPs was determined to be +15.6± 2.17 mV, indicating the polymeric nanoparticles stability.
    e. **Surface morphology of the developed formulation was observed with SEM and TEM-** Optimized nanoparticles were seeming to be nearly spherical with a smooth surface and dispersed evenly with minimal aggregation. The TEM picture clearly shows that most of the emulsion particles in the optimized formulation were less than 250 nm in size and spherical.
    f. **FTIR analysis, XRD studies and DSC Studies-** FTIR of freeze-dried optimized SL-P NPs revealed mostly the Eudragit RL 100 absorption peaks, with a few overlapping drug peaks. The peak, though, was not seen in the DSC curves derived from silymarin-piperine nanoparticles. The lack of endothermic peaks indicates that silymarin and piperine were present in NPs in a non-crystalline condition. The XRD of lyophilized powder of SL-P NPs was not shown any strong peak at the same position which indicated that both SL and P were in an amorphous state in polymeric nanoparticles.
    g. In vitro release studies- The raw silymarin, raw piperine, and Silybon-70 showed immediate release profiles of 94± 2.51%, 72.55 ± 3.22%, and 99.91± 3.48% in 9hr, 12 hrs., and 3 hrs. respectively. But in the case of optimized SL-P NPs, the release profile was found to be slow and sustained with 96.16± 2.87% in 24hrs
3. **Antioxidant potential of SL-Piperine NPs**

The results showed that the antioxidant effect of SL-P NPs was higher as compared to raw drug and Vitamin E as the piperine and silymarin combinedly give a potentiating effect to the prepared NPs.

4. In-vivo hepatotoxicity and biochemical analysis

When compared to normal control mice, high dose N-acetyl-p-amino-phenol intoxication mice group and empty nanoparticles along with N-acetyl-p-amino-phenol administration mice group. The combination of SL-P NPs provided stronger protection from N-acetyl-p-amino-phenol -induced hepatotoxic injury than either alone in the case of market product and silymarin-Piperine.

Recommendations of 2nd DSRC	**Compliance of 2nd DSRC (write clearly what changes were done)**
In vitro release studies and pharmacodynamic studies are recommended.	In vitro release studies and pharmacodynamic studies were performed.

Recommendations of URAC after 2nd DSRC	**Compliance**
In the executive summary the term Silymarin-piperine nanoparticles is abbreviated as SPNs and S-P-NPs. For effective writing the executive summary needs to be uniform in terms of correct was of writing abbreviated terms i.e., the abbreviation should be mentioned the first time it appears in the report and should be uniform throughout the document.	Uniform abbreviation is used throughout the document. The term Silymarin-piperine nanoparticles is abbreviated as SL-P NPs.

Recommendations of 3rd DSRC:

In vitro release studies have been carried out by using dialysis membrane number 60 (Hi Media) and compared with marketed formulation. Pharmacodynamic studies have been done on animal model as per the literature. Antioxidant studies have also been carried out.

Remaining work: Pharmacokinetic studies.

List of Publications (original research paper or Review), conference presentations and awards out of the PhD project (Proofs to be attached as annexure):

1. Anti-diabetic Effect of Sprouted Trigonella Foenum Graceum L. Seed Solid Dosage form in Low-dose Streptozotocin Induced Diabetic Rats **(Research)**
2. Characterization of Nano Phytopharmaceuticals **(Book Chapter)**
3. Comprehensive review on Liver Targeting: Emphasis on Nanotechnology Based Molecular Targets and Receptor mediated approaches **(Review)**
4. Autophagic Dysfunction in Dementia: Scope for Development of Potential Remedies **(Review)**
5. Characterization and HPLC Method Validation for Determination of Dopamine Hydrochloride in Prepared Nano Particles and Pharmacokinetic Application **(Research)**
6. Nano-delivery of Dietary Polyphenols for Therapeutic Applications **(Review)**
7. QbD Assisted Development and Validation of UV Spectroscopic Method in Estimation of Silymarin **(Research)**

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Executive Summary (4th DSRC)

Name of Research Scholar: Ashwini Kumar Mishra

Registration No.: 13/PhD/DPSRU/2019

Registration Date: 19th October 2019

Date of DSRC: 12 June 2023

Name of Supervisor: Prof. Pravat K. Sahoo

Title of Thesis: Formulation and Development of Dual-drug Loaded Nanoparticles of Silymarin with Natural Bioenhancer Against Paracetamol Induced Hepatotoxicity Mouse Model

Abstract of work done (300-500 words, in structured manner with separate paragraphs for Introduction ending with Objectives; Methodology; Results; Conclusions and proposed further work with expected outcomes):

Abstract of the thesis

Introduction with Objectives: In present study, natural product as bioenhancer is being selected based on its mechanism of action: Piperine – a known inhibitor of hepatic and intestinal glucuronidation inhibitor. This study will present enhanced liver cell targeting efficacy, absorption with long-acting drug formulation and to stablish its preparation method, particularly to a high-efficacy with increased bioavailability and improved absorption of silymarin by the virtue of piperine which is known for its bio-enhancing property. The technique nanoprecipitation will be adopted in this work in order to prepare a dual-drug loaded nanoparticles comprising of Silymarin and Piperine (a natural bioenhancer) for high-efficacy, long-acting formulation of silymarin characteristic of high bioavailability and smooth in vivo targeted release. Hepatoprotective and antioxidant potential of Silymarin-piperine nanoparticles (SL-P NPs) will be explored against Paracetamol induced hepatotoxicity in mouse.

Methodology: Characterization of the selected Drugs (Silymarin and piperine) was performed. Formulation and optimization of dual-drug loaded nanoparticles SL-P NPs by nanoprecipitation technique was done. Characterization of Optimized Formulation including Particle Size Analysis, PDI, Zeta Potential, entrapment efficiency of optimized SL-P NPs, surface morphology of the developed formulation was observed with SEM and TEM, FTIR,

XRD studies, DSC, in vitro release studies, antioxidant potential, *in-vivo* hepatotoxicity and biochemical analysis, histopathology, HPLC based pharmacokinetic study, stability study data of prepared formulation of optimized SL-P NPs were performed.

Results: The SL-P NPs were prepared in different batches in which concentrations of Eudragit RL100 and CTAB were varied at different rpm. The preparation method aids to synthesize NPs in a size range of 220-421 nm, having a PDI range between 0.138 to 0.331. The average size range of NPs varied from 220-421 nm, which was influenced by changing the concentration of ERL, CTAB, and stirring speed. PDI range was found to be in limits of 0.138-0.331 and ANOVA was performed to show the factors. The p-value (0.0001) less than 0.05 denotes the model terms as significant. Entrapment efficiency (%EE) of SL-P NPs was found to be in a range of 82.22-96.43%. Adjusted and predicted values of R^2 obtained were 0.989 and 0.980 respectively. The zeta potential of SL-P NPs was determined to be +15.6± 2.17 mV, indicating the polymeric nanoparticles stability. Optimized nanoparticles were seeming to be nearly spherical with a smooth surface and dispersed evenly with minimal aggregation. The TEM picture clearly shows that most of the emulsion particles in the optimized formulation were less than 250 nm in size and spherical. FTIR of freeze-dried optimized SL-P NPs revealed mostly the Eudragit RL 100 absorption peaks, with a few overlapping drug peaks. The peak, though, was not seen in the DSC curves derived from silymarin-piperine nanoparticles. The lack of endothermic peaks indicates that silymarin and piperine were present in NPs in a non-crystalline condition. The XRD of lyophilized powder of SL-P NPs was not shown any strong peak at the same position which indicated that both SL and P were in an amorphous state in polymeric nanoparticles. The raw silymarin, raw piperine, and Silybon-70 showed immediate release profiles of 94± 2.51%, 72.55 ± 3.22%, and 99.91± 3.48% in 9hr, 12 hrs., and 3 hrs. respectively. But in the case of optimized SL-P NPs, the release profile was found to be slow and sustained with 96.16± 2.87% in 24hrs. The results showed that the antioxidant effect of SL-P NPs was higher as compared to raw drug and Vitamin E as the piperine and silymarin combinedly give a potentiating effect to the prepared NPs. When compared to normal control mice, high dose Paracetamol intoxication mice group and empty nanoparticles along with Paracetamol administration mice group. The combination of SL-P NPs provided stronger protection from Paracetamol induced hepatotoxic injury than either alone in the case of market product and silymarin-Piperine. Histopathological examinations of mice liver portrayed the designed nanoparticulate formulation treatment of SL-P strongly protected mice against hepatotoxins and oxidative damage caused liver injury as the results showed minimal necrosis

and absence of foamy vacuolated cytoplasm in these groups as compared to Paracetamol treated group. The pharmacokinetic results showed that the $t_{1/2}$ of developed formulation is approximately 24 hours as compared to marketed which sustains for 12 hours. Stability study data of prepared formulation demonstrated that SL-P NPs were stable at 25 ±2℃ for 180 days with no significant change in particle size and entrapment efficiency. It can be concluded that the prepared SL-P NPs must be stored at temperature below 25℃ where they would remain stable in terms of PDI and zeta potential.

Conclusions and proposed work with expected outcomes: It would be a worthy statement toward the current research study that the increased antioxidant potential, enhanced bioavailability, and improved solubility were achieved when silymarin was used in combination with piperine incorporated in nano formulation. It was concluded that with better hepatoprotective potential inhibition of progression of liver diseases would be achieved.

Recommendations of 3rd DSRC	**Compliance of 3rd DSRC (write clearly what changes were done)**
In vitro release studies have been carried out by using dialysis membrane number 60 (Hi Media) and compared with marketed formulation. Pharmacodynamic studies have been done on animal model as per the literature. Antioxidant studies have also been carried out. Remaining work: Pharmacokinetic studies.	Pharmacokinetics studies were performed.

Recommendations of URAC after 3rd DSRC	**Compliance**
Publications related to thesis work are required. Name of drug “Acetaminophen” to be replaced with “Paracetamol.”	Publications related to thesis work are published and incorporated. Name of drug “Acetaminophen” has been replaced with “Paracetamol.”

Recommendations of 4th DSRC: Copy attached

Plagiarism report (Turnitin): Turnitin report of fourth DSRC report was attached as annexure.

List of Publications (original research paper or Review), conference presentations and awards out of the PhD project (Proofs to be attached as annexure):

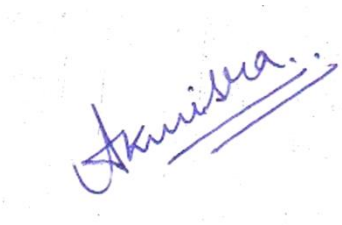
List of Publications (Title pages to be attached as annexure):

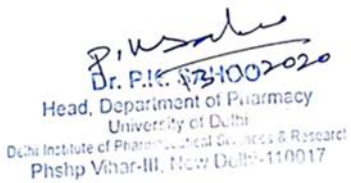
1. **Ashwini Kumar Mishra,** Neha SL, Laxmi Rani, Ankit Jain, Hitesh Kumar Dewangan, and Pravat Kumar Sahoo. Rationally Designed Nanoparticulate Delivery Approach for Silymarin with Natural Bio-Enhancer: In Vitro Characterization and in Vivo Evaluations of Hepatoprotective Effects in a Mouse Model. Journal of Drug Delivery Science and Technology (2023): 104580. https://doi.org/10.1016/j.jddst.2023.104580.**(I.F-5.06)**
2. Mithun Rudrapal, **Ashwini Kumar Mishra,** Laxmi Rani, Khomendra K Sarwa, James H Zothantluanga, Johra Khan, Mehnaz Kamal, Santwana Palai, Atul R Bendale, and Swati G Talele. Nanodelivery of Dietary Polyphenols for Therapeutic Applications. Molecules 27, no. 24 (2022): 8706. https://doi.org/10.3390/molecules27248706. **(I.F-4.927)**
3. **Ashwini Kumar Mishra,** Mukesh Pandey, Hitesh Kumar Dewangan, Neha SL, and Pravat Kumar Sahoo. A Comprehensive Review on Liver Targeting: Emphasis on Nanotechnology-Based Molecular Targets and Receptors Mediated Approaches. Current Drug Targets 23, no. 15 (2022): 1381-405. https://doi.org/10.2174/1389450123666220906091432. **(I.F-2.93)**
4. **Ashwini Kumar Mishra,** Neha SL, Laxmi Rani, Pravat Kumar Sahoo. QbD Assisted Development and Validation of UV Spectroscopic Method in Estimation of Silymarin, Letters in Drug Design & Discovery, DOI: 10.2174/1570180820666230126121026. **(I.F-1.09)**
5. **Ashwini Kumar Mishra,** Pravat Kumar Sahoo, Ganesh Lal, Meenakshi Bajpai, and Hitesh Kumar Dewangan. Anti-Diabetic Effect of Sprouted Trigonella Foenum-Graecum L. Seed Solid Dosage Form in Low-Dose Streptozotocin Induced Diabetic Rats. Indian Journal of Pharmaceutical Education and Research 56, no. 4 (2022): 1156-63. **(I.F- 0.686)**

6. **Ashwini Kumar Mishra,** Mukesh Pandey, Arzoo Pannu, Hitesh Kumar Dewangan, and Pravat Kumar Sahoo. Review on Diabetes Mellitus: An Insight into the Current Scenarios, the Challenges of Therapy, and Application of Traditional Drugs. Current Traditional Medicine 10 (2024): 1-22. https://dx.doi.org/http://dx.doi.org/10.2174/2215083810666230501212125. **(I.F-0.162)**
7. Neha S.L., **Ashwini Kumar Mishra,** Laxmi Rani, Suraj Pal Verma, Pravat Kumar Sahoo. Characterization and HPLC method validation for determination of dopamine hydrochloride in prepared nano particles and pharmacokinetic application, Analytical chemistry letters. 2022;12(4): 528-541.
8. **Ashwini Kumar Mishra** and Pravat Kumar Sahoo. "Characterization of Nano phytopharmaceuticals." Phyto-antioxidants and Nanotherapeutics (2022): 239-65. https://doi.org/10.1002/9781119811794.
9. Bhumika Kumar, Mukesh Pandey, **Ashwini Kumar Mishra** Anjali Sharma, Faizana Fayaz, and Faheem Hyder Pottoo. Autophagic Dysfunction in Dementia: Scope for Development of Potential Remedies. CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders) 20, no. 8 (2021): 704-22. https://doi.org/10.2174/1871527319666201209112256. **(I.F-2.82)**

List of conferences attended (Proofs to be attached as annexure): N/A

Patents Published / granted (Proofs to be attached as annexure): NA

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